Biotechnology Reports 39 (2023) e00808

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Research Article

Detergent-stable amylase production by *Paenibacillus lactis* strain OPSA3 isolated from soil; optimization by response surface methodology

Emmanuel Tobechukwu Ugwuoji [a,b], Tochukwu Nwamaka T. Nwagu [b,*], Lewis Iheanacho Ezeogu [b,c]

[a] *Department of Applied Microbiology and Brewing, Faculty of Biosciences, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria*
[b] *Department of Microbiology, Faculty of Biological Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria*
[c] *UNESCO International Centre for Biotechnology, Nsukka, Nigeria*

ARTICLE INFO

Keywords:
Detergent stable Amylase production
Waste valorisation
Response surface methodology
Green detergent
Paenibacillus

ABSTRACT

This study aimed to isolate thermostable, alkaliphilic, and detergent-tolerant amylase-producing bacteria. Pure isolates from environmental samples were screened on a starch-based medium (pH 11), and selected isolates were identified using cultural and molecular techniques. Product optimization studies were conducted, and secreted amylase was partially purified using 40% (w/v) saturation ammonium sulfate at 4 °C. The wash performance of concentrated amylase was analyzed. A novel isolate, Paenibacillus lactis OPSA3, was selected for further studies. The isolate produced amylase optimally when grown on banana peels and soybean extracts, which are agro-wastes. Optimization by Response surface Methodology resulted in a 2.1-fold increase in alkaliphilic amylase production. A 2.46-fold purification was achieved, with an enzyme activity yield of 79.53% and specific activity of 26.19 Umg^{-1}. Wash performance analysis using the amylase supplemented with boiled commercial detergent (kiln®) showed good cleaning efficiency. The amylase has the potential for application as a component of green detergent.

1. Introduction

Alpha amylase (E.C 3.2.1.1) catalyzes the hydrolysis of α-D-(1,4) glycosidic linkages in starch and related carbohydrates [1–3]. Amylases are among the most important enzymes used commercially, accounting for 25–33% of the world's total industrial enzyme market [4,5]. The hydrolytic action of amylases on starch is central to the industrial success of many biotechnological processes like starch processing, paper and pulp, textile, bioenergy, food, animal feed, detergent, fine chemicals and pharmaceuticals [1,4,6,7]. The future market for amylases is quite promising, with growing demands for processed foods, increased usage of amylases as emulsifier substitutes and functional additives in foods and beverages, and the detergent use of amylases as major drivers [8]. In their detergent use, amylases rank, by volume and value, second only to proteases [9,10], with application in dishwashing and in laundry, where they improve cleaning efficiency by removing starch-based soils and stains, such as sauces, pasta, potato, chocolate, custards and baby food, ice-creams and gravy [1,11,12]. Amylase use in detergent formulations is further reinforced by its full biodegradability, ensuring environmental safety [13,14]. Amylases also prevent swollen starch from adhering to the surface of laundry and dishes that may otherwise act as a glue for particulate soiling [15,16].

Detergent-compatible amylases must be alkaline pH-stable and active, possess long shelf lives, display oxidative stability and be stable and usable over a broad temperature range (e.g., 20–60 °C). It should also retain activity in the presence of the detergent's several other chemical components, like proteases, surfactants, bleaching agents and builders (calcium-chelating agents) (Paul et al., 2021; [17,18]). Bacterial enzymes, especially those from members of the class Bacilli, are often reported to possess the above qualities [13,19,20]. Members of the widely distributed genus *Paenibacillus* are well known for their elaboration of a wide range of biotechnologically relevant enzymes and enzyme systems, several of which are well active and stable under extreme environmental conditions, including those of pH and temperature [21–25]. Unfortunately, only a few reports exist of research on the detergent applicability of *Paenibacillus* sp. amylases [26–29].

One of the major challenges of the global enzyme industry is the high cost of enzyme production, owing to several factors, including the high

* Corresponding author.
E-mail address: tochukwu.nwagu@unn.edu.ng (T.N.T. Nwagu).

https://doi.org/10.1016/j.btre.2023.e00808
Received 20 April 2023; Received in revised form 13 July 2023; Accepted 16 July 2023
Available online 17 July 2023

Table 1
Actual factor levels corresponding to coded factor levels for central composite rotatable design of response surface methodology.

Variables	Code	Coded levels and their Actual Values				
		-α	−1	0	+1	+α
Banana Peel (%)	A	0.81821	1.5	2.5	3.5	4.18179
Spent Soybean Meal (%)	B	0	0.1	0.3	0.5	0.636359
Temperature (°C)	C	41.5910	45	50	55	58.4090

Table 2
Thermophilic and alkalophilic bacterial isolates obtained from the samples.

Samples	No of Isolates
Okpeye waste dump site soil (OPS)	10
Waste Paint dump Soil (PTS)	9
Garri/Cassava Processing waste soil (WPS)	12
Abattoir Waste Dumpsite soil (AWDS)	16
Salt lake Water (SLW)	6
Total	53

Table 3
Biochemical and morphological properties of OPSA3.

Test	Property
Gram Staining	+
Shape	Rods
Catalase	+
Oxidase	+
Indole	–
Methyl Red (MR)	–
Voges-Proskauer(VP)	+
Citrate	–
Nitrate Reduction	–
Motility	+
Enzymatic Reaction	
Phenylalanine deaminase	–
Casein Hydrolysis	–
Sugar Fermentation	
Xylose	–
Arabinose	+
Rhamnose	–
Glucose	+
Fructose	+
Galactose	–
Mannose	+
Trehalose	+
Sucrose	+
Lactose	+
Maltose	+
Starch	+
Mannitol	+
Glycerol	–

Keys: +=positive, - = negative.

cost of production of medium components [13]. An optimally constituted medium can engender marked increases in enzyme output and significantly lower production costs [30]. These benefits are further amplified when medium components come from cheap sources. Thus, highly nutrient-dense and valuable agro-industrial wastes like banana, cassava, potato peels, rice bran, and spent soybean meal can find use in the fermentation industry as medium components [31,32]. This is especially so in the enzyme industry, where increasing demands and high production costs have engendered research into using eco-friendly, low-cost, and easily available alternatives for enzyme production. Because of the prominent influence of fermentation media design over enzyme yield, agro-industrial wastes-based fermentation media must be optimized, alongside fermentation chemical and physical parameters, like medium pH, incubation temperature, inoculum size, chemical inhibitors, metal ions, etc., for fermentation practicability and economy [33,34]. Traditionally, fermentation process optimization had been carried out by the one-factor-at-a-time (OFAT) methodology [35]. Due to numerous shortcomings, chiefly its unsuitability for multifactor optimization, and its failure to provide reliable information on operational variables' interactions or enable accurate measurement of optimal experimental conditions, time-wasting, and high costs, OFAT is no longer popular. Statistical designs such as response surface methodology (RSM), Plackett-Burhman factorial design, Box-Behnken design, Taguchi experimental design approach, and central composite design (CCD), enabling one-time optimization of all process parameters are now preferred [36–38]. Several reports exist of successful RSM application, with methodologies like the Box-Behnken design, Plackett-Burhman factorial design, and central composite design (CCD), for process parameter optimization and attainment of enhanced enzyme production ([34,39–41]; Paul et al., 2021). RSM has been applied successfully for enhanced alpha-amylase production [4,42–46].

In the present study, we report the isolation and taxonomic identification of a high-titer and a detergent and surfactant stable alkaline amylase producing bacterial strain from disposal sites of Nigerian alkaline fermented condiment, *okpeye*. We also present the results of optimization studies for detergent-stable alkaline amylase production in submerged fermentation (SmF) using RSM and CCD. Finally, we characterized the partially purified enzyme for some of its biochemical properties, especially regarding its applicability in laundry detergent formulations for the cleaning industry.

2. Materials and experimental methods

2.1. Agro-industrial waste materials

Brewery spent grains (BSG) were obtained from Nigerian Brewery plc, 9th Mile Corner, Amah, Enugu State, Nigeria. Spent soybean meal, banana, potato and cassava peels, and rice bran were obtained from farmers and processors at Nsukka, in Enugu State, Nigeria. All agro-industrial waste materials were dried in a hot air oven at 60 °C for 24 h, then blended and filtered using a 200 mesh screen to fine powders.

2.2. Bacterial isolation and screening for amylase production

Soil samples (1.0 g) were suspended and homogenized in sterile distilled water (9 ml) before serial dilution. Aliquots (0.1 ml) of appropriately diluted samples (10^{-4} to 10^{-7} dilutions) were spread-plated on alkaline (pH 11.0) nutrient agar plates incubated, in an inverted position, at 50 °C, for 72 h. Primary (qualitative) screening for amylase production was with alkaline (pH 11.0) mineral salt starch medium (AMSSA) [47]. Zones of hydrolysis around bacterial colonies, observed after flooding with Gram's iodine (2% (w/v) I_2 and 0.2% (w/v) KI) solution, were measured and colonies with the largest clearing were selected. The secondary (quantitative) screening was by SmF. Bacterial inocula, 1 ml (10^6 CFU/ml), prepared by growing isolates at 50 °C, with shaking (150 rpm), to an absorbance (A_{600}) of 0.15 (equivalent to 10^6 CFU/ml), were transferred into sterile alkaline (pH 11.0) mineral salts broth (AMSSB), containing 3.0 g/L (w/) of starch. Growth was at 50 °C, for 48 h, in agitated (150 rpm) culture conditions. Cell-free culture supernatants, obtained after centrifugation (7800 × g, 15 min, at 25–32 °C), served as crude amylase. Isolates' detergent survivability was assayed according to Furmanczyk et al. [48], by growth on AMSSA with varying concentrations (0–5 μM) of sodium dodecyl sulfate (SDS).

Stocks of the purified cultures, grown on alkaline (pH 11.0) nutrient agar slants at 50 °C for 48 h, were preserved by storage in the fridge (4 °C).

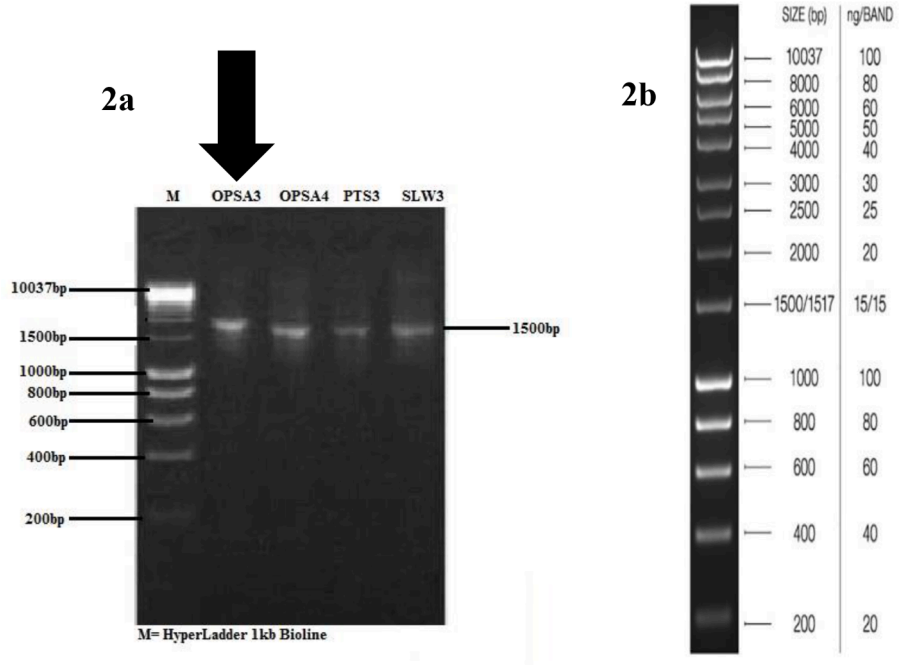

Fig. 1. Gel showing the size of PCR product (2a) and a hyper ladder1 (2b).

TACATGCAAGTCGAGCGGACTTGATGGAGTGCTTGCTCTCCTGATGGTTAGCGGCGGACGGGTGAGTAAC
ACGTAGGCAACCTGCCCTCAAGACTGGGATAACTACCGGAAACGGTAGCTAATACCGGATAATTAAATTC
GCTGCATGGCGGATTTATGAAAGGCGGAGCAATCTGTCACTTGAGGATGGGCCTGCGGCGCATTAGCTAG
TTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGAACGGCCACACTGGGA
CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACG
GAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGCCAGGGAAGAACGTCTCATA
GAGTAACTGCTATGAGAGTGACGGTACCTGAGAAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTCTTTAAGTCTG
GTGTTTAAACCCGAGGCTCAACTTCGGGACGCACTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGT
GGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGG
GCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGATGAATGCTAGGTGTTAGGGGTTTCGATACCCTTGGTGCCGAAGTTAACACATTAAGCATTCCG
CCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCAGTGGAGTATG
TGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAGTCTTGACATCCCTCTGAATCCTCTAGAGATAGA
GGCGGCCTTCGGGACAGAGGAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTT
AAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCACTTCGGGTGGGCACTCTAAGGTGACTGC
CGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACTTGGGCTACACACG
TACTACAATGGCTGGTACAACGGGAAGCGAAGCCGCGAGGTGGAGCCAATCCTAAAAAGCCAGTCTCAGT
TCGGATTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAATTGCTAGTAATCGCGGATCAGCATGCCGCGG
TGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTACAACACCCGAAGTCGGTG
GGGTAACCCTTACGGGAGCCAGCCGCCGAA

Fig. 2. The 16S rRNA sequence of the selected isolate, OPSA3 (1430 bp).

2.3. Microorganism and taxonomic study

Bacterial identification was detected by the (a) study of its phenotypic characteristics [49,50]; and (b) sequencing of the bacterial 16S rRNA gene. For the sequencing analysis, bacterial genomic DNA was isolated using the ZR fungal/bacterial DNA miniprep® kit (ZymoResearch as cat number: D6005) outlined by the kit manufacturer. PCR was performed for 30 cycles, each consisting of a 30 s denaturation step at 94 °C, a 90 s annealing step between 56 °C, and a 90 s extension step at 72 °C. Initial denaturation was at 94 °C for 5 min. The bacterial universal oligonucleotide primer universal oligonucleotide primers with the sequences; 27F: (5′ -AGAGTTTGATCMTGGCTCAG- 3′) and 1525R: (5′ – AAGGAGGTGWTCCARCCGCA- 3′), were used. PCR products were purified and directly used for DNA sequencing on 3130 Genetic Analyzer (Applied Biosystem, Switzerland). The deduced sequence was subjected to the BLAST search tool from the National Center of Biotechnology, Bethesda, MD (http://www.ncbi.nlm.nih.gov) to retrieve the homologous sequences in GenBank. The above sequences can be retrieved from the GenBank using accession number MW600272.

2.3.1. Phylogenetic analysis

Gene sequence data were aligned with reference sequences from the NCBI database using the multiple sequence alignment program, MEGA X [51–53]. The construction of phylogenetic trees was by distance matrix-based cluster algorithms, viz. unweighted pair group method with averages (UPGMA), neighbor-joining, maximum likelihood, and maximum-parsimony analysis as described elsewhere [51]. All ambiguous positions were removed for the sequence pair. The stability of trees obtained from the above cluster analysis was assessed using the BOOTSTRAP program in sets of 1000 re-samplings (MEGA 4).

2.4. Amylase production studies

The effects of process parameters on alkaline amylase production in

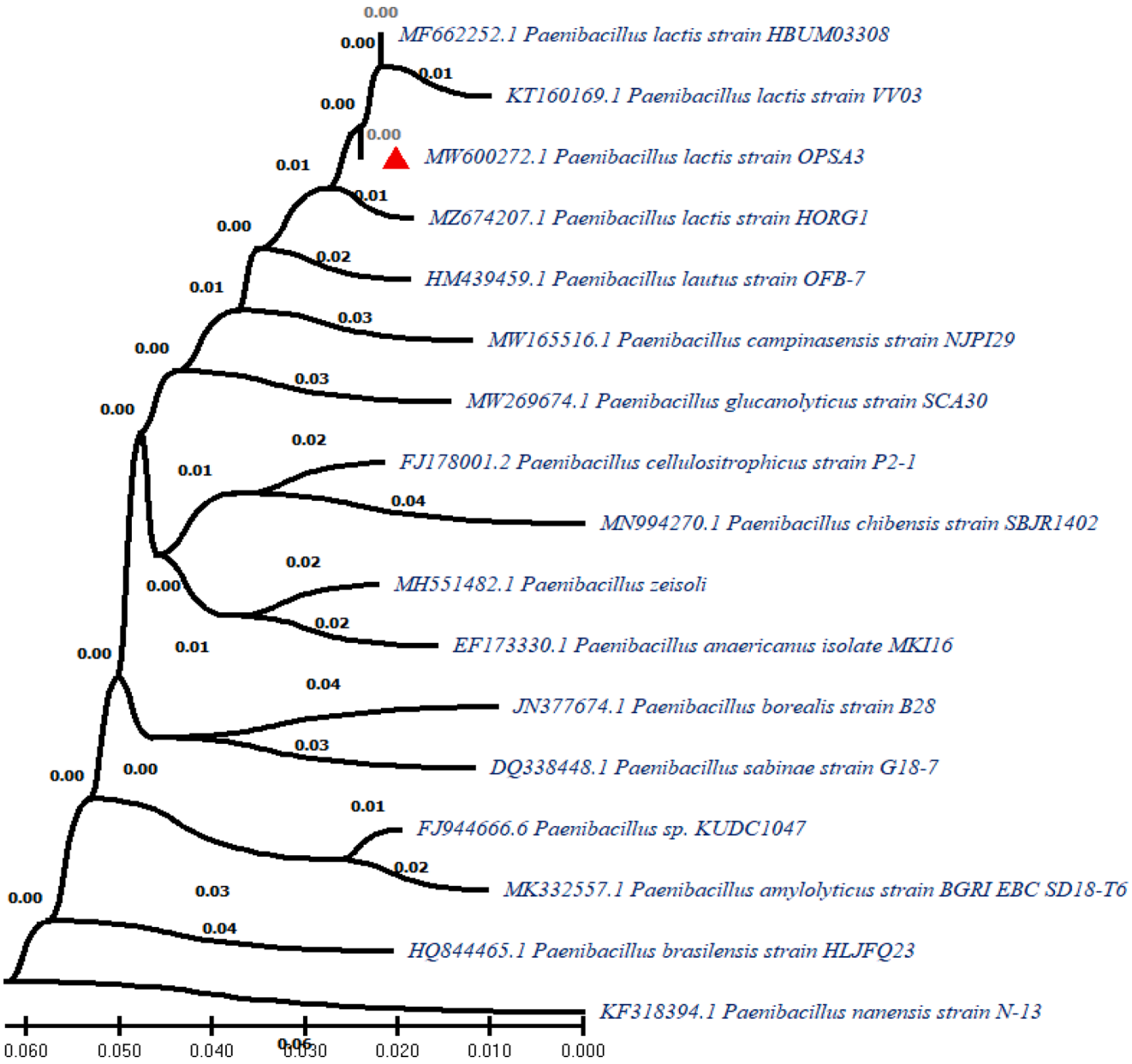

Fig. 3. Phylogenetic relationship of strain OPSA3 to other closely related strains of *Paenibacillus lactis* and species of *Paenibacillus*.

submerged fermentation (SmF) were studied. Fermentation medium comprised of (g/l): carbon sources, 25.0; spent soybean meal, 6.0; $NaNO_3$, 5.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; NaCl, 0.5; $CaCl_2$, 0.01, and KCl, 0.5. Medium pH was adjusted to 11.0. Sterile medium (50 ml) in 250 ml Erlenmeyer flask was inoculated with 24 h nutrient broth culture of isolate (adjusted to 10^6 CFU/ml) and incubated in a rotary shaker at 50 °C and 150 rpm for 72 h. Samples were collected every 6 h for analysis. For the effects of C-sources (2.5% (w/v)), brewery spent grain, banana peels, potato peels, rice bran, cassava peels, and soluble starch were studied. For inorganic nitrogen sources, $(NH_4)_2SO_4$ and NH_4NO_3, as well as $NaNO_3$, were studied. Cow blood meal, yeast extract, malt extract and soybean meal comprised the organic nitrogen sources investigated.

2.4.1. Estimation of amylase activity

The reducing sugars released by crude amylase (0.5 ml) action on 1.0% (w/v) soluble starch (0.5 ml, in 50 mM glycine-NaOH buffer, pH 9.0), at 50 °C, for 10 min was measured using 3,5-dinitrosalicylic acid (DNS) reagent [54]. The absorbance determination was at 540 nm against an enzyme blank. One unit of amylase activity was defined as any amount of enzyme releasing 1 μmol of glucose equivalent per minute from soluble starch under assay conditions. All assays were performed in triplicates.

2.4.2. Estimation of protein

Protein was determined according to the Lowry et al. [55] method using BSA (bovine serum albumin) as standard.

2.5. Statistical optimization

Levels of the significant parameters and the effects of interactions among the fermentation medium's various constituents, which significantly influenced amylase production, were analyzed and optimized by RSM. A five-level central composite design (CCD) (-α, −1, 0, +1 and +α) was used (Table 1) was used to study the optimum concentration and interactions of significant process (independent) variables, including (i) concentration (% w/v) of C-source (X1); (ii) concentration (% w/v) of N-sources (X2); (iii) temperature (°C) of growth (X3). Using the Minitab

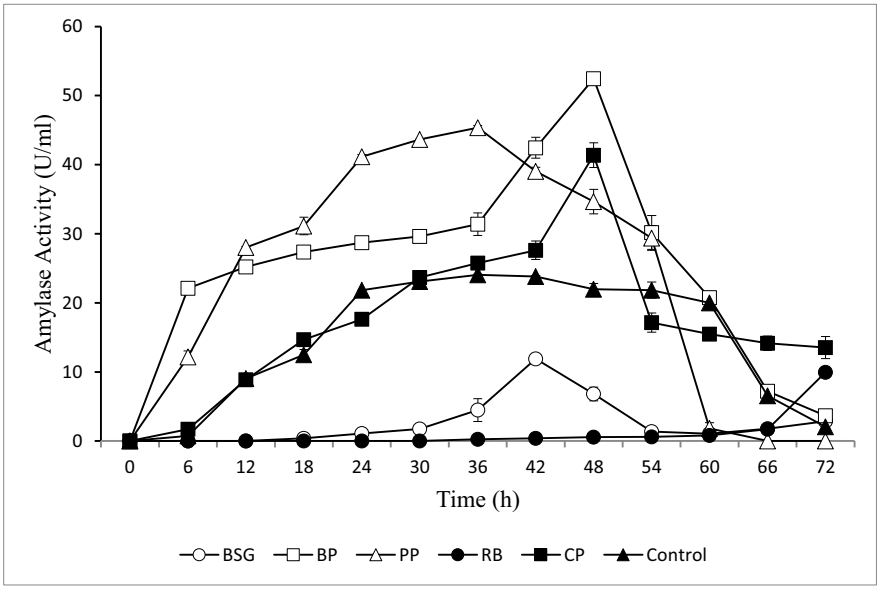

Fig. 4. Effect of C-source on time-course of amylase production by isolate *P. lactis* OPSA3.

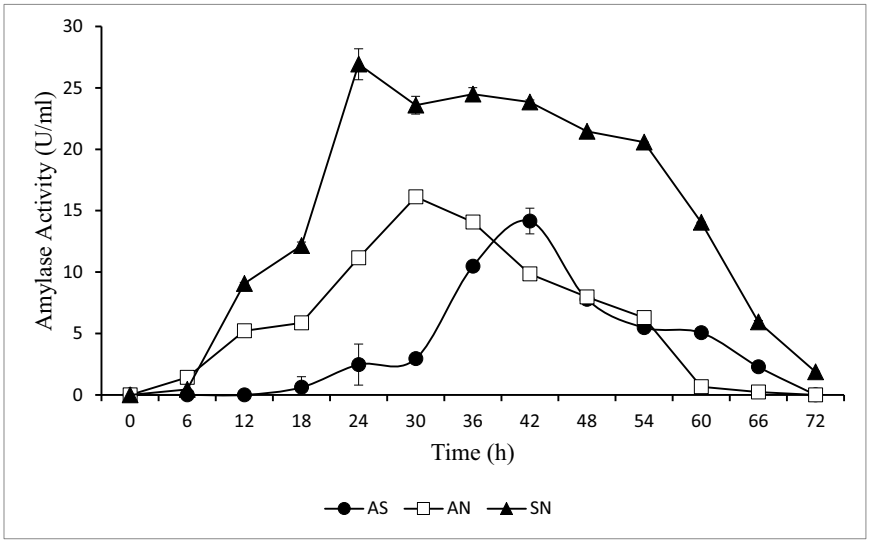

Fig. 5. Effect of inorganic N-source on time-course of amylase production by isolate *P. lactis* OPS A3.

17 Statistical Software® (Minitab Inc., PA, USA), a set of 20 randomized experiments was generated and run in triplicates. The average enzyme activity values were taken as the dependent variable or response/output. Fermentation was under agitated culture conditions (150 rpm) for 48 h.

2.5.1. Statistical analysis and modeling

CCD data were subjected to analysis of variance (ANOVA) and multiple regression analysis, performed by fitting first order polynomial equations, including individual and cross effect of each variable. The result (Eq. (1)) was an empirical model relating response measured in the independent variable to the experiment. The model equation was:

$$Y = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_4X_1X_2 + B_5X_1X_3 + B_6X_2X_3 + B_7X_1^2 + B_8X_2^2 + B_9X_3^2 \quad (1)$$

where, B0–B9 are the regression coefficients of the respective variables and interaction terms computed from the observed experimental values of measured response. X1–X3 are the codes for independent variables. The measured responses, Y = amylase activity (U/ml), with constrains applied are described in Table 2.1. Responses of variables were analyzed by three-dimensional plots.

2.5.2. Validation of the experimental model

The optimized condition achieved during RSM was adopted to design a ten (10) run validation experiment. Enzyme production was carried out under conditions (medium C- and N-source concentrations) predicted by the model to give optimal enzyme production. Fermentation was carried out at different temperatures (45 °C, 50 °C and 55 °C), for 48 h, at 150 rpm. Samples were collected and amylase determined as earlier described.

2.6. Production and partial purification of amylase

Amylase was produced in batch SmF in the RSM-optimized medium, composed of 25.0 g/L banana peel and 6.0 g/l spent soybean meal at 50 °C, pH 11.0. All other cultivation conditions were as previously described. Cell-free supernatant, prepared by centrifugation (7800 x g, 15 min, 25–30 °C) of 48 h post-inoculation culture, served as the crude

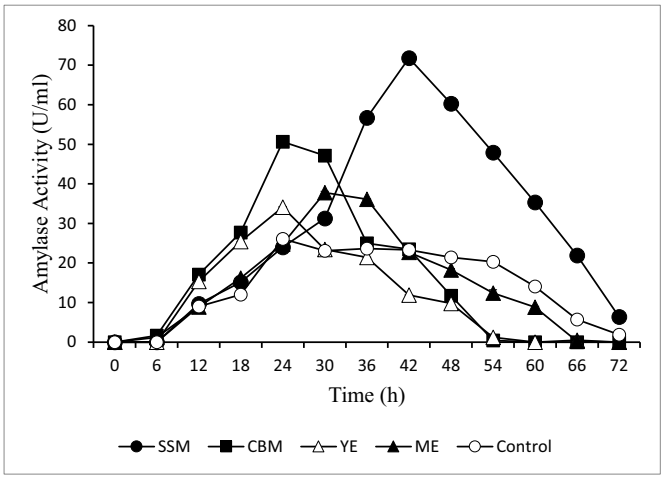

Fig. 6. Effect of organic N-source on time-course of amylase production by isolate *P. lactis* OPS A3.

enzyme. Solid ammonium sulfate was added, slowly and with gentle stirring, to 1000 ml of the cell-free supernatant, at 4 °C to attain 40% (w/v) saturation. The precipitated protein, dissolved in a minimal volume of 20 mM sodium phosphate buffer (pH 8.0), was dialysed extensively, 16 h, at 4 °C, against distilled water to remove the salt [56].

2.7. Analysis of amylase compatibility with commercial laundry detergents and wash performance

The wash performance analyses of the concentrated amylase were according to Bhange et al. [13] and Dahiya and Rathi [20]. Stain removal ability was assessed using white cotton cloth (6 cm x 6 cm) soiled with tomato ketchup (100 µl) and dried overnight at room temperature and in an oven (60 °C) for two h to enable firm attachment of the stain to the fabric. Stained cloth pieces were subjected to washing, in a shaker (200 rpm), at 50 °C, for 1 h, using four different wash treatments, as follows: water only (treatment 1); water and boiled detergent (Klin®) (7 mg/ml) (treatment 2); water and amylase (treatment 3); and, water with commercial boiled detergent (Klin®) (7 mg/ml) supplemented with 1 ml of partially purified amylase (treatment 4). After treatment, the clothes were rinsed in clean water (20 ml) before drying (1 h, 25–30 °C). Untreated cloth pieces stained with tomato ketchup served as control. Cleaning performance was visually observed, and then pictures were taken.

3. Results and discussion

3.1. Isolation, screening and identification

Thermophilic and alkalophilic bacteria can be isolated from different parts of the environment. From the various samples collected, 53 distinct bacteria colonies were isolated (Table 2). The amylase screening on starch agar plates confirmed that most isolates were amylase producers. Based on the diameter of starch hydrolysis on the agar plates, ten isolates were selected and tested for the ability to secrete amylase in submerged culture. Seven of these were isolated from *okpeye* waste dumpsite soil. OPSA3 produced the highest amylase (45.78 ± 2.67 U/ml), followed by OPSA4 with an amylase yield of 35.10 ± 1.22 U/ml. Further analysis showed that OPSA3 exhibited a higher tolerance (2.0 µM) to detergent (SDS) than the others from different environmental sources. Based on the highest amylase secretion and tolerance to SDS, OPSA3 was selected for further studies.

OPSA3 is a Gram-positive, spore-forming, motile, rod-shaped, catalase-positive and oxidase positive vegetative cell (Table 3). On nutrient agar, it presented as transparent to milky, extensively branched colonies with tip-splitting. On starch agar plates, the colonies were slightly elevated, smooth, and whitish, with irregular margins. It grew luxuriantly on nutrient and starch agar at 50 °C and pH 11.0. OPSA3 was positive for VP but negative for MR, nitrate reductase, citrate utilization and indole production tests. It utilized starch and a range of sugars.

PCR amplification of strain OPSA3's 16S rRNA gene generated a unique band on the gel (Fig. 1) of 1425 bp (Fig. 2). The 16S rRNA-based phylogenetic analysis of OPSA3 shows that this isolate shared 94.5% with Paenibacillus lactis. Isolate OPSA3 probably represents a new strain of P. lactis. Its 16S rRNA gene sequence has been registered in the NCBI Genbank, with accession number MW600272. A 16SrRNA-based phylogenetic tree reflecting the relatedness of P. lactis OPSA3 to other Paenibacillus species and sister P. lactis strains is shown in Fig. 3. Like many other members of the Firmicutes Class Bacilli, members of the genus Paenibacillus have been isolated from a confoundingly wide variety of environmental niches, including different types of soils [57–61],

Table 4
Actual and predicted values of amylase activity with the experimental runs obtained through CCD based RSM.

Run Order	Std. Order	Pt. Type	Banana Peel (% w/v)	Spent Soybean Meal (% w/v)	Temperature (°C)	Predicted Amylase Activity (U/ml)	Actual Amylase Activity (U/ml)
1	6	1	3.50	0.10	55.00	64.03	56.43±2.65
2	8	1	3.50	0.50	55.00	99.22	94.41±3.09
3	1	1	1.50	0.10	45.00	28.73	21.57±1.29
4	3	1	1.50	0.50	45.00	33.41	29.05±1.77
5	7	1	1.50	0.50	55.00	25.58	17.34±0.66
6	10	−1	4.18	0.30	50.00	77.84	84.91±0.66
7	9	−1	0.81	0.30	50.00	0.03	0.83±0.00
8	4	1	3.50	0.50	45.00	88.59	83.15±2.43
9	16	0	2.50	0.30	50.00	96.62	92.64±1.77
10	5	1	1.50	0.10	55.00	15.91	9.39±1.99
11	15	0	2.50	0.30	50.00	96.62	89.55±0.88
12	18	0	2.50	0.30	50.00	96.62	102.14±0.22
13	17	0	2.50	0.30	50.00	96.62	86.46±0.44
14	19	0	2.50	0.30	50.00	96.62	103.02±0.22
15	11	−1	2.50	0.00	50.00	61.67	70.78±1.55
16	20	0	2.50	0.30	50.00	96.62	103.01±0.21
17	12	−1	2.50	0.63	50.00	95.19	103.02±1.99
18	14	−1	2.50	0.30	58.40	49.12	59.52±1.77
19	2	1	3.50	0.10	45.00	58.39	54.66±1.33
20	13	−1	2.50	0.30	41.59	50.99	57.53±3.32

Table 5
Analysis of Variance (ANOVA) generated by response surface full quadratic model for optimization of amylase production.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	21,045.6	2338.40	22.73	0.000
Linear	3	10,469.2	3489.74	33.93	0.000
Banana Peel	1	9108.4	9108.45	88.55	0.000
Spent Soybean Meal	1	1356.6	1356.64	13.19	0.005
Temperature	1	4.1	4.13	0.04	0.845
Square	3	10,067.9	3355.95	32.62	0.000
Banana Peel* Banana Peel	1	6972.5	6972.49	67.78	0.000
Spent Soybean Meal* Spent Soybean Meal	1	595.8	595.81	5.79	0.037
Temperature* Temperature	1	3905.4	3905.42	37.97	0.000
2-Way Interaction	3	508.6	169.52	1.65	0.240
Banana Peel* Spent Soybean Meal	1	325.8	325.78	3.17	0.105
Banana Peel* Temperature	1	170.4	170.37	1.66	0.227
Spent Soybean Meal* Temperature	1	12.4	12.42	0.12	0.735
Error	10	1028.6	102.86		
Lack-of-Fit	5	748.7	149.73	2.67	0.152
Pure Error	5	280.0	56.00		
Total	19	22,074.3			

R^2 - 95.34%.

compost [62], fermented soybeans [63,62], Spanish style green olive fermentation [64], alcohol fermentation [21] and Pu'er tea fermentation [65]. Several members of the genus promote crop growth directly through biological nitrogen fixation, phosphate solubilization, indole-3-acetic acid (IAA) production, and the release of siderophores that enable iron acquisition [66]. The association of this species with the production of plant growth-promoting traits (such as phosphorus solubilization) [67,68] and the isolation of Paenibacillus prosopidis, a related member of the genus, from root nodules of a plant relative of P. africana, Prosopis farcta (Syrian mesquite) [69], indicates the possibility that P. lactis may constitute a part in P. africana's natural microbiome. This probably also explains its presence in and isolation from okpeye waste dumpsite in this study.

Paenibacillus contains some of the best-known carbohydrate-active enzyme (CAZyme)-producing bacteria, like P. polymyxa, P. amylolyticus and Paenibacillus thiaminolyticus [70–73]. Despite previous reports of amylase production by Paenibacillus species, the current one, to the best of our knowledge, is the first to associate a strain of P. lactis with some extracellular amylase production.

Okpeye fermentation, like that of the other traditional fermented protein seeds (e.g., daddawa, ugba, etc.), is essentially alkaline, involving mostly members of the Firmicutes, especially of the class Bacilli, often resulting in products with pH $\geq$ 8.5 [74]. Alkaline vegetable protein seed fermentations have, thus, served as good sources for the isolation of alkaliphilic thermophilic bacteria. P. polymyxa (the Paenibacillus type species), an alkaliphilic and thermophilic member, has previously been isolated from ogiri (Citrullus vulgaris) fermentation [75,76]. The thermophilic and alkaliphilic nature of P. lactis OPSA3 from wastes of the alkaline fermented okpeye corresponds with previous isolations of alkaliphilic and thermophilic bacteria of the Class Bacilli (e.g. B. subtillis natto [77], B. amyloliquefasciens [78], B. circulans [79,80], Brevibacillus bortelensis [81,82] from alkaline fermented vegetable seed proteins.

3.2. Production studies

Medium components are important determinants of enzyme productivity by bacteria [40]. Under SmF conditions, P. lactis OPSA3 produced amylases using cheap agro-industrial wastes, banana peel and soybean meal as carbon and nitrogen sources, respectively, at 50 °C. The C-source remarkably influenced amylase production by the P. lactis OPSA3 (Fig. 4), with banana peel eliciting the highest amylase yield (52.45 ± 0.50 U/ml) compared to potato peel (45.35 ± 0.24 U/ml), cassava peel (41.38±1.26 U/ml), brewery spent grain (11.91±0.45 U/ml), and rice bran (9.96±0.51 U/ml). Maximum amylase elaboration with banana occurred after 48 h of growth. All inorganic N-sources supported amylase production (Fig. 5), although enzyme synthesis was significantly ($p < 0.05$) affected by the nature of the inorganic nitrogen source. **$NaNO_3$** elicited the highest amylase production (26.92±0.89 U/ml), followed by ammonium nitrate (16.13±0.19 U/ml). $(NH_4)_2SO_4$ gave the lowest amylase yield (14.15±0.74 U/ml). Amylase activity was detectable after only 12 h of fermentation, irrespective of the inorganic N-source used. The inorganic N source led to a variation of the optimum production time ($p < 0.05$), especially with $NaNO_3$, with peak enzyme activity attained after 48 h. The nature of the organic nitrogen source was also a significant ($p < 0.05$) determinant of enzyme production level, with peak amylase activity (71.78±0.12 U/ml) achieved using spent soybean meal (Fig. 6). Similar to C- and inorganic N-sources, the organic N- source significantly influenced ($p < 0.05$) the time-course profile of amylase production by P. lactis OPS A3. The peak amylase production was attained after 42, 24, 24, and 30 h of fermentation with spent soymeal, cow blood meal, and yeast extract, respectively.

Many studies have reported using expensive carbon and nitrogen sources (e.g., malt extract, yeast extract, beef extract) in the formulation of media for industrial amylase production [41,83,84]. Using inexpensive, renewable media components to produce industrial microbial enzymes will ensure that production processes are much more sustainable and cost-effective. Successful production of amylase by Paenibacillus lactis OPSA3, while growing on cheap agricultural waste resources, strongly agrees with the results of other studies [85–88], reporting successful use of agricultural waste materials (banana peel, cassava peel, spent grain, potato peel, rice bran, wheat bran) in fermentation media formulation for microbial enzyme and metabolite production. Furthermore, because these agricultural waste materials are often common pollutants, being frequently discarded wrongfully in the environment, their successful use in industrial fermentation media formulations could contribute markedly to environmental sustainability [85].

3.3. Statistical optimization

Appropriate designing of fermentation media is vitally central to the optimal performance and commercial success of industrial fermentation processes [89,40]. Preference for the use of RSM, over conventional experimental approaches, in media optimization, for enzyme production stems from two critical factors, the first being the latters' need for numerous separate experiments to determine the influence of each factor on enzyme production. The second factor is the inability of conventional methods to provide information on the effects of media factor

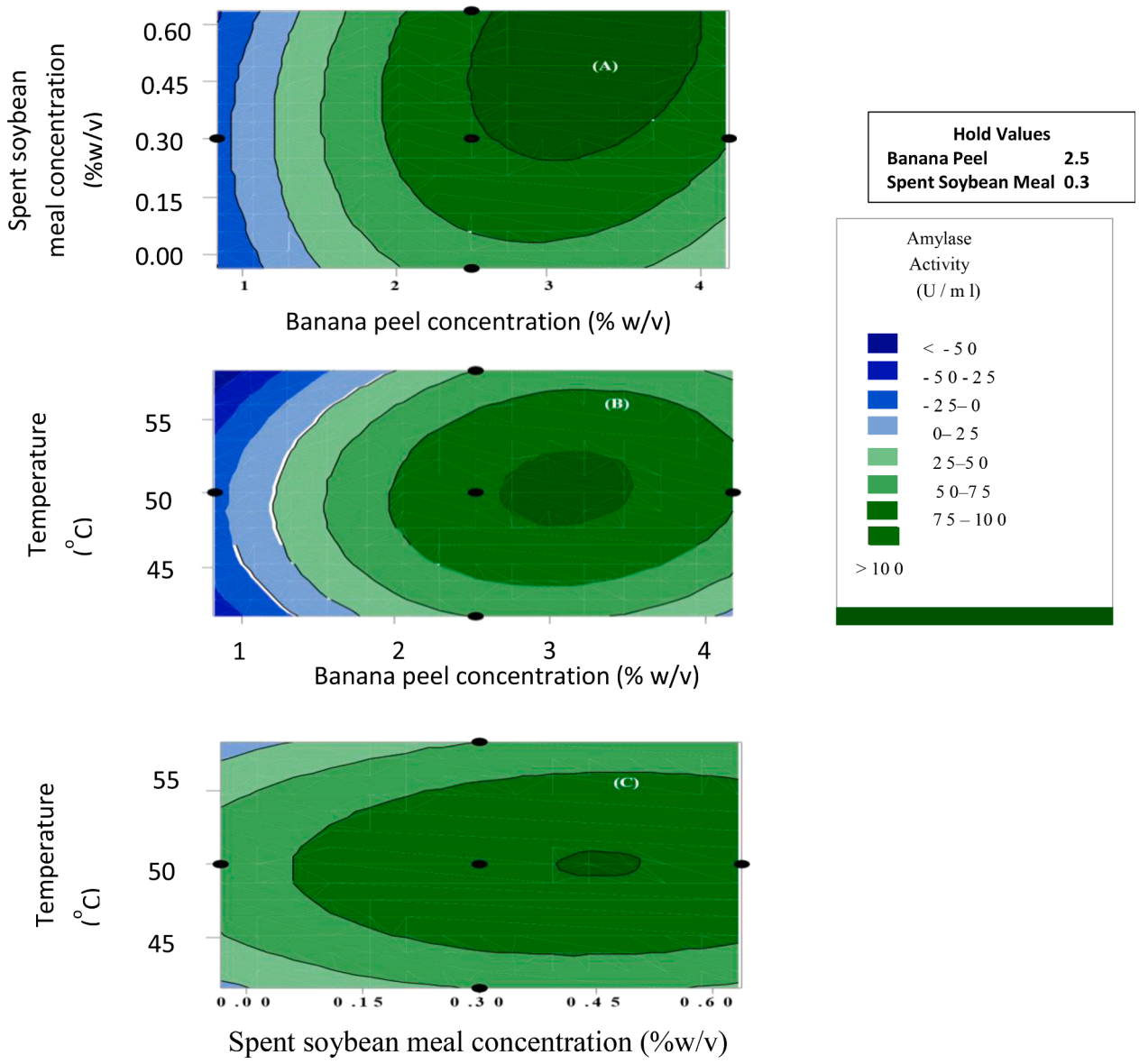

Fig. 7. Contour plots of alkaline amylase production by *Paenibacillus lactis* strain OPSA3.

interactions on enzyme production. Based on the results of initial screening processes, the importance of three test variables, banana peel, spent soybean meal and temperature, on alkaline amylase production by P. lactis, we proceeded to optimize these parameters using RSM involving central composite design (CCD) as represented in Table 4. Multiple regression analysis was used to analyze the data; thus, a polynomial equation that best expresses the nature of the relationship existing between Paenibacillus lactis OPSA3 alkaline amylase production and the studied factors was derived from a regression analysis as follows:

$$AA\ (U/ml) = -1617 + 80.1\ BP + 4\ SSM + 63.1T - 22BP^2 - 160.7SSM^2 - 0.658T^2 + 31.9(BP * SSM) + 0.923(BP * T) + 1.25(SSM * T) \quad (2)$$

where, AA is alkaline amylase, BP and SSM represent the concentrations (g) of banana peel and spent soybean meal, respectively, and T refers to fermentation temperature (°C).

From the Eq. (2), it is quite apparent that all the terms of the model, including T (temperature, °C), BP (concentration,% w/v of banana peel), and SSM (concentration,% w/v of spent soybean meal), with all their pair-wise interactions, significantly ($p < 0.05$) influenced alkaline amylase production. Banana peel and spent soybean meal concentrations and temperature positively influenced ($p < 0.05$) amylase development, as did each of their 2-way interactions. On the other hand, each of the variables' respective squares negatively impacted enzyme production ($p < 0.05$).

The significance of each regression coefficient was evaluated statistically using ANOVA (Table 5). The P-value for the model and "lack-of-fit" values were 0.00 and 0.152, respectively, suggesting a good fit between obtained experimental data and the statistical model. The observed R2 value for the multiple correlations for observed/actual alkaline amylase activity indicates that the fitted model can explain

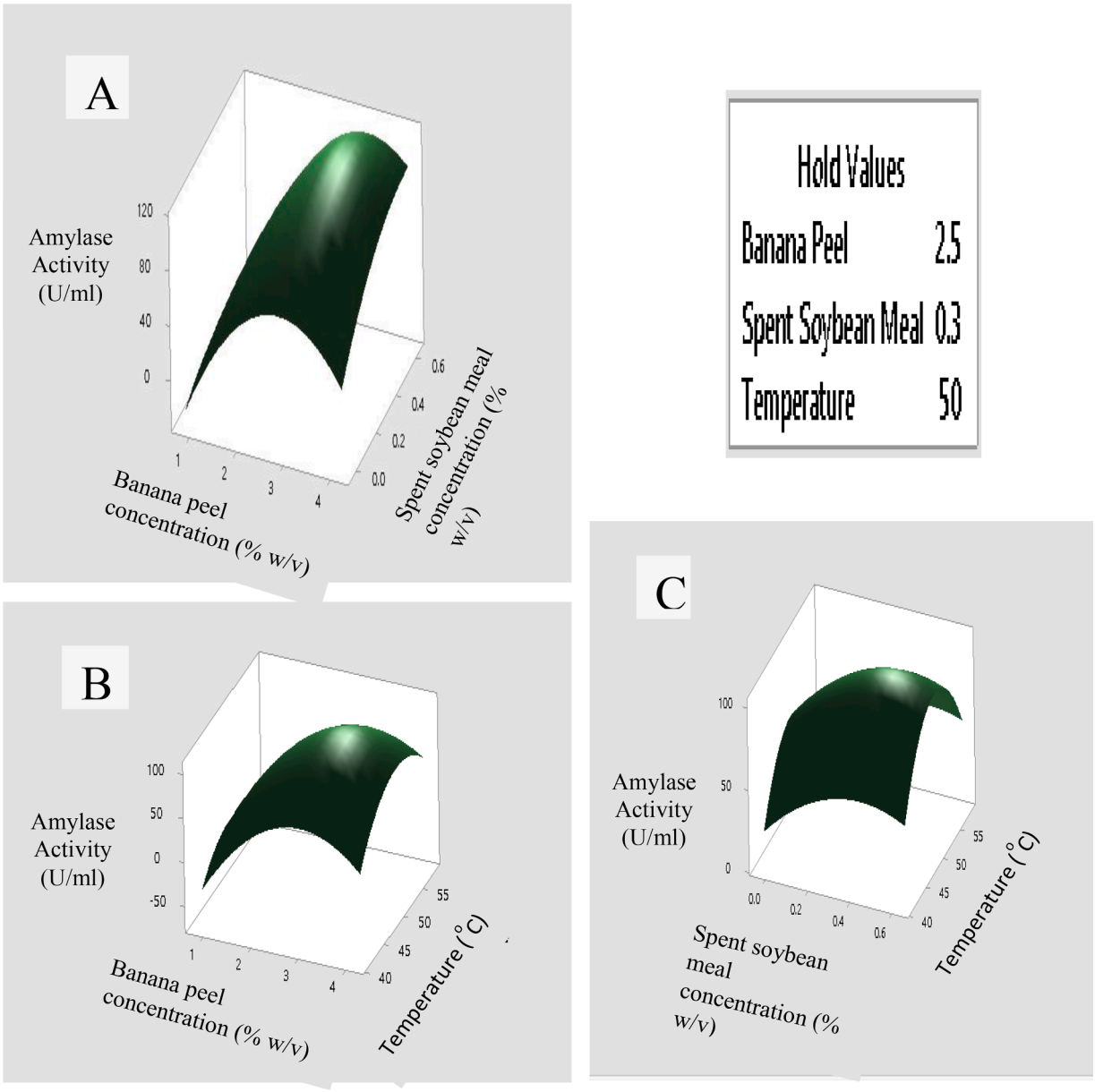

Fig. 8. Surface plots of alkaline amylase production by *Paenibacillus lactis* strain OPSA3.

Table 6
Actual and predicted values of amylase activity (U/ml) with the experimental run obtained through RSM based validation experiment.

Exp. No	Banana Peel (%w/v)	Spent Soybean Meal (%w/v)	Temperature (°C)	Predicted (U/ml)	Observed (Actual) (U/ml)
1	4.0	0.4	45	70.57	78.47±8.41
2	2.5	0.5	50	100.16	93.92±5.26
3	2.5	0.6	45	79.32	80.67±5.26
4	2.0	0.4	50	79.99	82.88±2.10
5	3.0	0.5	50	110.76	118.21±2.10
6	2.5	0.5	45	83.00	85.99±7.79
7	2.5	0.5	55	84.39	78.46±2.10
8	1.5	0.5	50	45.96	32.09±5.26
9	4.0	0.3	50	85.87	85.09±5.26
10	3.0	0.1	55	68.49	69.63±6.31

95.34% of the total variation. This strongly vouches for the adequacy of the model.

Response surface curves were plotted to determine the interaction effects and optimal levels of the variables, including contour (Fig. 7) and surface plots (Fig. 8).

Three-dimensional surface and contour plots graphically present the regression equation and facilitate understanding of the interactions of independent variables while helping to locate the optimum level of each

Table 7
Summary of the partial purification of alkaline amylase of *Paenibacillus lactis* OPSA3.

Purification	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Activity yield (%)
Crude (cell-free) extract	111.29	10.48	10.62	1.00	100.00
Dialysate enzyme	88.52	3.38	26.19	2.46	79.53

variable for maximum response [90]. The surface and contour plots showed the response for the interactive factors of banana peel, spent soybean meal and temperature when the other medium components, including mineral salts, inorganic nitrogen source, incubation time, pH, and inoculum concentration, were kept constant. All three response surface plots were convex-shaped, indicating well-defined optimum variables [91]. Similarly, all three contour plots were elliptically shaped, showing strong evidence of interactions between the different variables [92]. Increasing banana peel and spent soybean meal concentrations to 3% and 0.5%, respectively, and keeping fermentation temperature at 50 °C were optimal for alkaline amylase production. Above these concentrations, a reduction in enzyme production was observed. Overall, statistical optimization of the above process variables, using RSM, resulted in 2.1 folds more alkaline amylase production by P. lactis OPSA3. This value was higher than the corresponding value of 0.84-fold reported for Bacillus subtilis PF1 amylase by Bhange et al. [13] after RSM optimization of a medium consisting of agro-industrial waste materials.

The calculations projected enzyme production by P. lactis OPSA3 to be highest in culture medium containing banana peel and spent soybean meal at 3.0 and 0.5% (w/v), respectively, at 50 °C. Model validation results (Table 6) show that the predicted values for alkaline amylase were in good agreement with values obtained in the optimized medium. This optimized condition improved alkaline amylase production from 56.29±1.83 U/ml to 118.21±2.10 U/ml.

3.4. Partial purification of amylase

The amylase was partially purified from crude enzyme by ammonium sulfate precipitation. An approximately 2.5 purification fold with a yield in enzyme activity of 79.53% and a specific activity of 52.38 U/mg was recorded. The partial purification results have been summarized (Table 7). These results are higher than the 1.86 purification fold, 9.33 U/mg specific activity and 63.00% yield recorded by Obafemi et al. [93] using amylase from *Aspergillus niger*. Almost similarly, Bhaskara Rao et al. [94] reported a partially purified amylase from *Bacillus* sp. *marini* that exhibited a specific activity of 0.016 U/ml/mg which corresponds to 2.66 purification fold and 70.5% yield. Tallapragada et al. [95] reported 6.46 fold purification and a specific activity of 0.446 U/mg using $(NH_4)_2SO_4$ precipitation 60% saturation for an amylase of *Monascus sanguineus*. Another study, using the alpha amylase of *Bacillus subtilis* (MTCC 121), reported approximately 3-fold increase in specific activity compared to the crude [96].

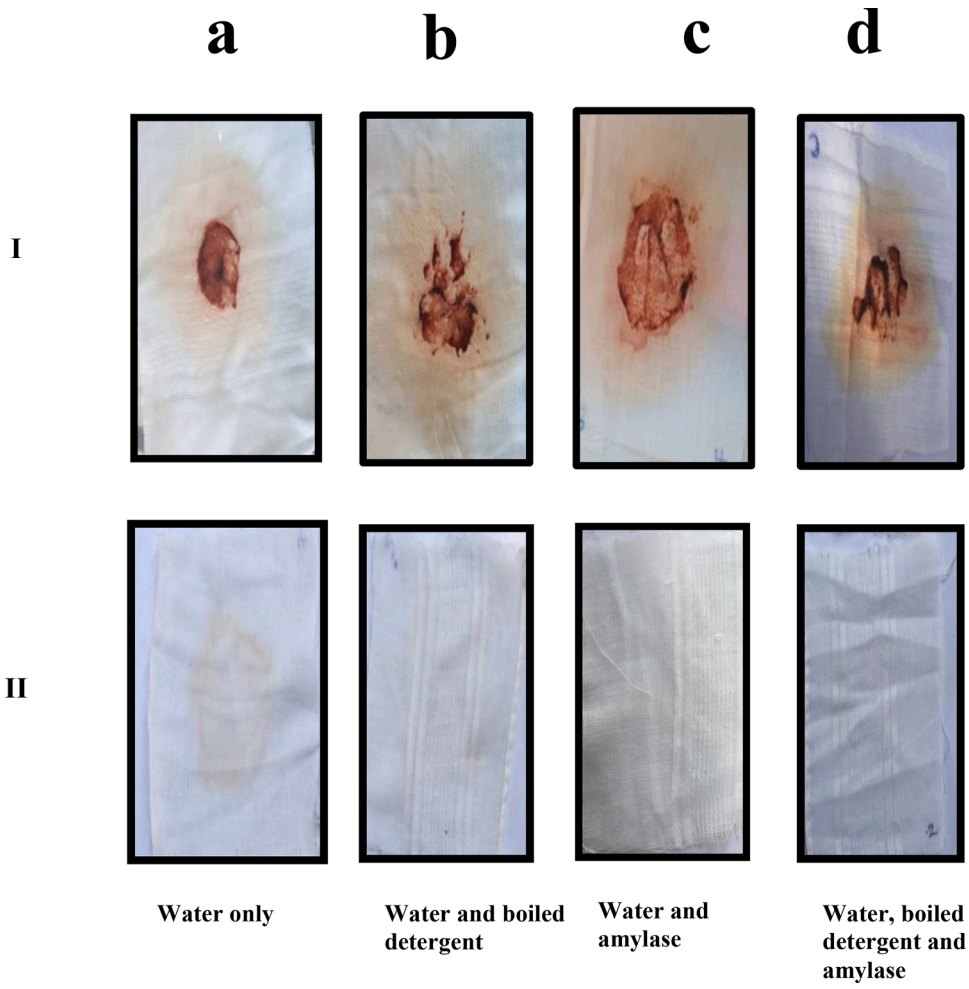

Fig. 9. Washing performance evaluation of the amylase from Paenibacillus *lactis* strain OPSA3 in the presence of the commercial detergent, Klin®. (a) Cloth stained with tomato ketchup washed with tap water; (b) tomato ketchup-stained cloth washed with tap water and boiled klin detergent (7 mg/mL), (c) tomato ketchup-stained cloth washed with tap water and partially purified amylase, (d) tomato ketchup-stained cloth washed with tap water, boiled klin detergent and partially purified amylase I: untreated cloths (control) and II: treated cloths.

3.5. Wash performance analysis

A mixture consisting of water, P. lactis OPSA3 alkaline amylase and boiled commercial detergent removed the stain from ketchup-stained cotton fabrics the most, compared to water alone, water plus boiled detergent, or only water plus amylase (Fig. 9). From the pictures, it is evident that treatment with P. lactis OPSA3 alkaline amylase enhanced the cleaning efficiency of the boiled detergent. Dahiya and Rathi [20] reported similar enhancement of fabric cleansing properties (potato curry stains) of detergents by bacterial amylases from Bacillus licheniformis MTCC1483. In the current study, our results indicate the compatibility of P. lactis OPSA3 alkaline amylase with commercial detergents of varying formulations. This finding highlights a strong potential for the future application of P. lactis OPSA3 alkaline amylase as an additive in laundry detergent formulations.

4. Conclusion

This study reports the successful isolation and characterization of Paenibacillus lactis OPSA3, a novel alkalophilic bacterium secreting a thermophilic, detergent-stable, alkaline amylase from okpeye waste dumpsite soil. Alkaline amylase production by P. lactis OPSA3 was markedly dependent on the carbon and nitrogen source in the fermentation medium. However, using cheap, locally abundant agro-waste material in medium formulation elicited the highest alkaline amylase production, highlighting its immense potential in fermentation medium formulation for sustainable and economical industrial bioprocessing. Optimization of process parameters with RSM, including experimental design, regression and model generation, resulted in an optimized medium with 110% more alkaline amylase yield from P. lactis OPSA3. Wash performance analysis confirmed P. lactis OPSA3 alkaline amylase as detergent-stable, enhancing the cleansing ability of various commercial laundry detergents. The results of this study make a case for the future laundry detergent application of P. lactis OPSA3 alkaline amylase.

CRediT authorship contribution statement

Emmanuel Tobechukwu Ugwuoji: Data curation, Formal analysis, Visualization, Writing – original draft. **Tochukwu Nwamaka T. Nwagu:** Conceptualization, Methodology, Supervision, Project administration, Writing – review & editing. **Lewis Iheanacho Ezeogu:** Project administration, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data has been made available in this work.

References

[1] N.A. Al-Dhabi, G.A. Esmail, A.K.M. Ghilan, M.V. Arasu, Isolation and screening of *Streptomyces sp.* Al-Dhabi-49 from the environment of Saudi Arabia with concomitant production of lipase and protease in submerged fermentation, Saudi J. Biol. Sci. 27 (1) (2020) 474–479, https://doi.org/10.1016/j.sjbs.2019.11.011.

[2] S.A. Mohamed, A.L. Al-Malki, T.A. Kumosani, Partial purification and characterization of five α-amylases from a wheat local variety (balady) during germination, Aust. J. Basic Appl. Sci. 3 (2009) 1740–1748.

[3] T.N. Nwagu, H. Aoyagi, B.N. Okolo, Adsorption and stabilization of a raw starch digesting amylase on microbead silica gel 300A, Br. Biotechnol. J. 2 (2) (2012) 85–101.

[4] B. Bhatt, V. Prajapati, K. Patel, U. Trivedi, Kitchen waste for economical amylase production using *Bacillus amyloliquefaciens* KCP2, Biocatal. Agric. Biotechnol. 101654 (2020), https://doi.org/10.1016/j.bcab2020.101654.

[5] A. Deljou, I. Arezi, Production of thermostable extracellular α-amylase by a moderate thermophilic *Bacillus licheniformis* isolated from Qinarje Hot Spring (Ardebil prov. of Iran), Period. Biol. 118 (4) (2017), https://doi.org/10.18054/pb.v118i4.3737.

[6] T.N. Nwagu, B.N. Okolo, H. Aoyagi, Immobilization of raw starch digesting amylase on silica gel: a comparative study, Afr. J. Biotechnol. 10 (71) (2011) 15989–15997.

[7] T.N. Nwagu, B.N. Okolo, H. Aoyagi, Stabilization of raw-starch digesting amylase by multipoint covalent attachment of glutaraldehyde-activated amberlite beads, J. Microbiol. Biotechnol. 22 (5) (2012) 628–636.

[8] Lucintel, 2020. Amylase market: trends, forecast and competitive analysis. Retrieved from https://www.lucintel.com/amylase-market.aspx.

[9] A. Hammami, N. Fakhfakh, O. Abdelhedi, M. Nasri, A. Bayoudh, Proteolytic and amylolytic enzymes from a newly isolated *Bacillus mojavensis* SA: characterization and applications as laundry detergent additive and in leather processing, Int. J. Biol. Macromol. 108 (2018) 56–68.

[10] F.N. Niyonzima, S.S. More, Detergent-compatible bacterial amylases, Appl. Biochem. Biotechnol. 174 (2014) 1215–1232, https://doi.org/10.1007/s12010-014-1144-3.

[11] C. Pundir, N. Chauhan, Coimmobilization of detergent enzymes onto a plastic bucket and brush for their application in cloth washing, Ind. Eng. Chem. Res. 51 (9) (2012) 3556–3563, https://doi.org/10.1021/ie202053r.

[12] R. Vidyalakshmi, R. Paranthaman, J. Indhumathi, Amylase production on submerged fermentation by *Bacillus spp*, World J. Chem. 4 (2009) 89–91.

[13] K. Bhange, V. Chaturvedi, R. Bhatt, Simultaneous production of detergent stable keratinolytic protease, amylase and biosurfactant by *Bacillus subtilis* PF1 using agro industrial waste, Biotechnol. Rep. 10 (2016) 94–104, https://doi.org/10.1016/j.btre.2016.03.007.

[14] S. Das, S. Singh, V. Sharma, M.L. Soni, Biotechnological applications of industrially important amylase enzyme, Int. J. Pharm. Biol. Sci. 2 (1) (2011) 486–496.

[15] S. Mitidieri, A.H. Souza Martinelli, A. Schrank, M.H. Vainstein, Enzymatic detergent formulation containing amylase from *Aspergillus niger*: a comparative study with commercial detergent formulations, Bioresour. Technol. 97 (10) (2006) 1217–1224, https://doi.org/10.1016/j.biortech.2005.05.02.

[16] H.S. Olsen, P. Falholt, The role of enzymes in modern detergency, J. Surfactants Deterg. 1 (4) (1998) 555–567, https://doi.org/10.1007/s11743-998-0058-7.

[17] R. Gupta, P. Gigras, H. Mohapatra, V.K. Goswami, B. Chauhan, Microbial α-amylases: a biotechnological perspective, Process Biochem. 38 (11) (2003) 1599–1616, https://doi.org/10.1016/S0032-9592(03)00053-0.

[18] S. Priyadarshini, S.K. Pradhan, P. Ray, Production, characterization, and application of thermostable, alkaline α-amylase (AA11) from *Bacillus cereus* strain SP-CH11 isolated from Chilika Lake, Int. J. Biol. Macromol. 145 (2020) 804–812, https://doi.org/10.1016/j.ijbiomac.2019.11.149.

[19] T.L.R. Corrêa, S.K. dos Santos Moutinho, M.L.L. Martins, M.A. Martins, Simultaneous α-amylase and protease production by the soil bacterium *Bacillus sp.* SMIA-2 under submerged culture using whey protein concentrate and corn steep liquor: compatibility of enzymes with commercial detergents, Food Sci. Technol. 31 (4) (2011) 843–848, https://doi.org/10.1590/s0101-20612011000400003 (Campinas).

[20] P. Dahiya, B. Rathi, Characterization and application of alkaline α-amylase from *Bacillus licheniformis* MTCC1483 as a detergent additive, Int. Food Res. J. 22 (3) (2015) 1294–1298.

[21] L. Chen, L. Wang, X.F. Sheng, *Paenibacillus qingshengii* sp. nov., isolated from a lead–zinc tailing, Int. J. Syst. Evol. Microbiol. 65 (2015) 2161–2166.

[22] X.F. Huang, F.Z. Wang, W. Zhang, J. Li, J. Ling, J. Yang, *Paenibacillus abyssi* sp. nov., isolated from an abyssal sediment sample from the Indian Ocean, Antonie Van Leeuwenhoek 106 (2014) 1089–1095. International Journal of General and Molecular Microbiology.

[23] M.J. Montes, E. Mercadé, N. Bozal, J. Guinea, *Paenibacillus antarcticus* sp. nov., a novel psychrotolerant organism from the Antarctic environment, Int. J. Syst. Evol. Microbiol. 54 (2004) 1521–1526.

[24] M. Orencio-Trejo, S. De la Torre-Zavala, A. Rodriguez-Garcia, H. Aviles-Arnaut, A. Gastelum-Arellanez, Assessing the performance of bacterial cellulases: the use of *Bacillus* and *Paenibacillus* strains as enzyme sources for lignocellulose saccharification, Bioenergy Res. 9 (4) (2016) 1023–1033, https://doi.org/10.1007/s12155-016-9797-0.

[25] Q.Y. Tang, N. Yang, J. Wang, Y.Q. Xie, B. Ren, Y.G. Zhou, *Paenibacillus algorifonticola* sp. nov., isolated from a cold spring, Int. J. Syst. Evol. Microbiol. 61 (2011) 2167–2172.

[26] T. Rajesh, Y.H. Kim, Y.K. Choi, et al., Identification and functional characterization of an α-amylase with broad temperature and pH stability from *Paenibacillus sp*, Appl. Biochem. Biotechnol. 170 (2013) 359–369, https://doi.org/10.1007/s12010-013-0197-z.

[27] K. Tsusaki, H. Watanabe, T. Yamamoto, T. Nishimoto, H. Chaen, S. Fukuda, Purification and characterization of highly branched α-Glucan–producing enzymes from *Paenibacillussp. PP710*, Biosci. Biotechnol. Biochem. 76 (4) (2012) 721–731, https://doi.org/10.1271/bbb.110855.

[28] I. Ul-Haq, U. Hameed, Z. Mahmood, M.M. Javed, Solid state fermentation for the production of a-amylase by *Paenibacillus amylolyticus*, Pak. J. Bot. 44 (2012) 341–346.

[29] I.K.L. Ul-Haq, U. Hameed, Z. Mahmood, M.M. Javed, Solid State Fermentation for the production of alpha-amylase by *Paenibacillus amylolyticus*, Pak. J. Bot. 44 (2012) 341–346.

[30] D. Gangadharan, S. Sivaramakrishnan, K.M. Nampoothiri, R.K. Sukumaran, A. Pandey, Response surface methodology for the optimization of alpha amylase production by *Bacillus amyloliquefaciens*, Bioresour. Technol. 99 (2008) 4597–4602, https://doi.org/10.1016/j.biortech.2007.07.028.

[31] S.A. Mohamed, A.L. Al-Malki, J.A. Khan, S.A. Kabli, S.M. Al-Garni, Solid state production of polygalacturonase and xylanase by *Trichoderma species* using cantaloupe and watermelon rinds, J. Microbiol. 51 (2013) 605–611.

[32] R. Singh, R. Das, S. Sangwan, Utilisation of agro-industrial waste for sustainable green production: a review, Environ. Sustain. (2021), https://doi.org/10.1007/s42398-021-00200-x.

[33] Du, S.S., Zhai, X., Poczos, B., Singh, A., 2018. Gradient descent provably optimizes over-parameterized neural networks. ICLR (conference paper). [cs.LG].

[34] N. Srivastava, M. Srivastava, B.D. Malhotra, V.K. Gupta, P.W. Ramteke, R.do N. Silva, et al., Nanoengineered cellulosic biohydrogen production via dark fermentation: a novel approach, Biotechnol. Adv. 37 (6) (2019) 13, https://doi.org/10.1016/j.biotechadv.2019.04.006.

[35] V. Singh, S. Haque, R. Niwas, A. Srivastava, M. Pasupuleti, C.K.M. Tripathi, Strategies for fermentation medium optimization: an in-depth review, Front. Microbiol. 7 (2017), https://doi.org/10.3389/fmicb.2016.02087.

[36] K. Kumar, S. Srivastav, V.S. Sharanagat, Ultrasound Assisted Extraction (UAE) of bioactive compounds from fruit and vegetable processing by-products: a review, Ultrason. Sonochem. (2020), 105325, https://doi.org/10.1016/j.ultsonch.2020.10532.

[37] M.F.B. Majid, H.F.B.M. Zaid, C.F. Kait, K. Jumbri, L.C. Yuan, S. Rajasuriyan, Futuristic advance and perspective of deep eutectic solvent for extractive desulfurization of fuel oil: a review, J. Mol. Liq. 112870 (2020), https://doi.org/10.1016/j.molliq.2020.112870.

[38] € M.S. Tanyildizi, M. Elibol, D. Ozer, Optimization of growth medium for the production of a-Amylase from *Bacillus amyloliquefaciens* using response surface methodology, J. Chem. Technol. Biotechnol. 81 (2006) 618–622.

[39] Z.K. Bagewadi, S.I. Mulla, H.Z. Ninnekar, Response surface methodology based optimization of keratinase production from *Trichoderma harzianum* isolate HZN12 using chicken feather waste and its application in dehairing of hide, J. Environ. Chem. Eng. 6 (4) (2018) 4828–4839, https://doi.org/10.1016/j.jece.2018.07.007.

[40] J.K. Roy, S.K. Rai, A.K. Mukherjee, Characterization and application of a detergent-stable alkaline alpha-amylase from *Bacillus subtilis* strain AS-S01a, Int. J. Biol. Macromol. 50 (1) (2012) 219–229.

[41] A.A. Simair, A.S. Qureshi, I. Khushk, C.H. Ali, S. Lashari, M.A. Bhutto, G. S. Mangrio, C. Lu, Production and partial characterization of A-Amylase enzyme from *Bacillus Sp.* BCC 01- 50 and potential applications, BioMed Res. Int. 2017 (2017) 1.

[42] S.A. Ahmed, M.A. Abdella, G.M. El-Sherbiny, A.M. Ibrahim, A.R. El-Shamy, S. M. Atalla, Application of one–factor-at-a-time and statistical designs to enhance α-amylase production by a newly isolate *Bacillus subtilis* strain-MK1, Biocatal. Agric. Biotechnol. 22 (2019), 101397, https://doi.org/10.1016/j.bcab.2019.101397.

[43] F. Allala, K. Bouacem, N. Boucherba, S. Mechri, M. Kriaa, W. Arkoub-Djoudi, Z. Azzouz, S. Benallaoua, H. Hacene, B. Jaouadi, A. Bouanane-Darenfed, α-amylase production by *Tepidimonas fonticaldi* strain HB23: statistical optimization and compatibility study for use in detergent formulations, Environ. Sci. Pollut. Res. Int. 27 (29) (2020) 37164–37172, https://doi.org/10.1007/s11356-020-10142-2.

[44] M. Kherouf, A. Habbeche, F. Benamia, B. Saoudi, B. Kerouaz, A. Ladjama, Statistical optimization of a novel extracellular alkaline and thermostable amylase production from thermophilic *Actinomadura keratinilytica* sp. Cpt29 and its potential application in detergent industry, Biocatal. Agric. Biotechnol. 35 (2021), 102068, https://doi.org/10.1016/j.bcab.2021.102068.

[45] M.J. Msarah, I. Ibrahim, A.A. Hamid, W.S. Aqma, Optimisation and production of alpha amylase from thermophilic *Bacillus spp*. and its application in food waste biodegradation, Heliyon 6 (6) (2020) e04183, https://doi.org/10.1016/j.heliyon.2020.e04183.

[46] P.Y. Stergiou, A. Foukis, L. Theodorou, M. Papagianni, E. Papamichael, Optimization of the production of extracellular α-amylase by *Kluyveromyces marxianus* IF0 0288 by response surface methodology, Braz. Arch. Biol. Technol. 57 (3) (2014) 421–426, https://doi.org/10.1590/s1516-8913201401485.

[47] P. Kaur, A. Vyas, Characterization and optimal production of alkaline αamylase from *Bacillus sp*. DLB 9, Afr. J. Microbiol. Res. 6 (11) (2012) 2674–2681. http://www.academicjournals.org/AJMR.

[48] E.M. Furmanczyk, M.A. Kaminski, G. Spolnik, M. Sojka, W. Danikiewicz, A. Dziembowski, L. Lipinski, A. Sobczak, Isolation and characterization of Pseudomonas spp. strains that efficiently decompose sodium dodecyl sulfate, Front. Microbiol. 8 (2017) 1872, https://doi.org/10.3389/fmicb.2017.01872.

[49] T. Hussain, A. Roohi, S. Munir, I. Ahmed, J. Khan, V. Edel-Hermann, K.Y. Kim, M. Anees, Biochemical characterization and identification of bacterial strains isolated from drinking water sources of Kohat, Pakistan, Afr. J. Microbiol. Res. 7 (16) (2013) 1579–1590, https://doi.org/10.5897/AJMR12.2204.

[50] M.D. Libman, M. Kramer, R. Platt, Comparison of Gram and Kopeloff stains in the diagnosis of bacterial vaginosis in pregnancy, Diagn. Microbiol. Infect. Dis. 54 (2006) 197–201.

[51] S. Kumar, G. Stecher, M. Li, C. Knyaz, K. Tamura, MEGA X: molecular evolutionary genetics analysis across computing platforms, Mol. Biol. Evol. 35 (2018) 1547–1549.

[52] N. Saitou, M. Nei, The neighbor-joining method: a new method for reconstructing phylogenetic trees, Mol. Biol. Evol. 4 (1987) 406–425.

[53] K. Tamura, M. Nei, S. Kumar, Prospects for inferring very large phylogenies by using the neighbor-joining method, in: 101, 2004, pp. 11030–11035.

[54] P. Bernfeld, Amylases α and β methods. Methods in Enzymology. USA, Academic Press, New York, 1955, pp. 149–158.

[55] O.H. Lowry, N.J. Rosebrough, A.L. Farr, et al., Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–273.

[56] S. Priyadarshini, P. Ray, Exploration of detergent-stable alkaline α-amylase AA7 from *Bacillus sp* strain SP-CH7 isolated from Chilika Lake, Int. J. Biol. Macromol. 140 (2019) 825–832.

[57] J.H. Ahn, B.C. Kim, B.Y. Kim, S.J. Kim, J. Song, S.W. Kwon, *Paenibacillus cucumis* sp. nov. isolated from greenhouse soil, J. Microbiol. 52 (2014) 460–464.

[58] S.J. Cho, S.H. Cho, T.S. Kim, S.H. Park, S.B. Kim, G.H. Lee, *Paenibacillus insulae* sp. nov., isolated from soil, J. Microbiol. 53 (2015) 588–591.

[59] B. Hungund, V.S. Shyama, P. Patwardhan, A.M. Saleh, Production of polyhydroxyalkanoate from *Paenibacillus durus* BV-1 isolated from oil mill soil, J. Microb. Biochem. Technol. 5 (2013) 13–17.

[60] H.S. Kim, S. Srinivasan, S.S. Lee, *Paenibacillus alba* nov., isolated from peat soil, Curr. Microbiol. 70 (2015) 865–870.

[61] J. Tang, S. Qi, Z. Li, Q. An, M. Xie, B. Yang, Production, purification and application of polysaccharide-based bioflocculant by *Paenibacillus mucilaginosus*, Carbohydr. Polym. 113 (2014) 463–470.

[62] I. Vaz-Moreira, C. Faria, M.F. Nobre, P. Schumann, O.C. Nunes, C.M. Manaia, *Paenibacillus humicus* sp. nov., isolated from poultry litter compost, Int. J. Syst. Evol. Microbiol. 57 (2007) 2267–2271.

[63] H. Tsumori, A. Shimamura, Y. Sakurai, K. Yamakami, Substrate specificity of mutanase of *Paenibacillus humicus* from fermented food, J. Health Sci. 57 (2011) 78–81.

[64] H. Lucena-Padrós, B. Caballero-Guerrero, A. Maldonado-Barragán, J.L. Ruiz-Barba, Microbial diversity and dynamics of Spanish-style green table-olive fermentations in large manufacturing companies through culture-dependent techniques, Food Microbiol. 42 (2014) 154–165.

[65] B.C. Kim, K.H. Lee, M.N. Kim, E.M. Kim, M.S. Rhee, O.Y. Kwon, *Paenibacillus pinihumi* sp. nov., a cellulolytic bacterium isolated from the rhizosphere of *Pinus densiflora*, J. Microbiol. 47 (2009) 530–535.

[66] E.N. Grady, J. MacDonald, L. Liu, A. Richman, Z. Yuan, Current knowledge and perspectives of *Paenibacillus*: a review, Microb. Cell Factory 15 (2016) 203, https://doi.org/10.1186/s12934-016-0603-7.

[67] L. Andrades-Moreno, I. Del Castillo, R. Parra, B. Doukkali, S. Redondo-Gómez, P. Pérez-Palacios, M.A. Caviedes, E. Pajuelo, I.D. Rodríguez-Llorente, Prospecting metal-resistant plant-growth promoting rhizobacteria for rhizoremediation of metal contaminated estuaries using *Spartina densiflora*, Environ. Sci. Pollut. Res. Int. 21 (5) (2014) 3713–3721, https://doi.org/10.1007/s11356-013-2364-8.

[68] D. Goswami, J.N. Thakker, P.C. Dhandhukia, Portraying mechanics of plant growth promoting rhizobacteria (PGPR): a review, Cogent Food Agric. 2 (2016) 1, https://doi.org/10.1080/23311932.2015.1127500.

[69] A. Valverde, A. Fterich, M. Mahdhi, M.H. Ramírez-Bahena, M.A. Caviedes, M. Mars, *Paenibacillus prosopidis* sp. nov., isolated from the nodules of *Prosopis farcta*, Int. J. Syst. Evol. Microbiol. 60 (2010) 2182–2186.

[70] S. Dhawan, Purification of a Thermostable β-mannanase from Paenibacillus Thiaminolyticus-characterization and its potential use as a detergent additive, J. Pure Appl. Microbiol. 15 (1) (2021) 368–381, https://doi.org/10.22207/JPAM.15.1.31.

[71] S. Ghio, O. Ontañon, F.E. Piccinni, *Paenibacillus* sp. A59 GH10 and GH11 extracellular endoxylanases: application in biomass bioconversion, Bioenergy Res. 11 (2018) 174–190, https://doi.org/10.1007/s12155-017-9887-7.

[72] C. Keggi, J. Doran-Peterson, *Paenibacillus amylolyticus* 27C64 has a diverse set of carbohydrate-active enzymes and complete pectin deconstruction system, J. Ind. Microbiol. Biotechnol. 46 (1) (2019) 1–11, https://doi.org/10.1007/s10295-018-2098-1.

[73] L. Lincoln, V.S. More, S.S. More, Purification and biochemical characterization of extracellular glucoamylase from *Paenibacillus amylolyticus* strain, J. Basic Microbiol. (2019), https://doi.org/10.1002/jobm.201800540.

[74] C. Parkouda, D.S. Nielsen, P. Azokpota, L.I.R. Ouoba, W. Amoa-Awua, L. Thorsen, J.D. Hounhouigan, J.S. Jensen, K. Tano-Debrah, B. Diawara, M. Jakobsen, The microbiology of alkaline-fermentation of indigenous seeds used as food condiments in Africa and Asia, Crit. Rev. Microbiol. 35 (2) (2009) 139–156, https://doi.org/10.1080/10408410902793056.

[75] I.O.A. Jideani, C.R. Okeke, Comparative study of microorganisms and sensory attributes of condiments from the fermentation of different seeds, Plant Foods Hum. Nutr. 41 (1991) 27–34.

[76] B. Omafuvbe, O. Shonukan, S. Abiose, Microbiological and biochemical changes in the traditional fermentation of soybean for "soy-daddawa" — Nigerian food condiment, Food Microbiol. 17 (5) (2000) 469–474, https://doi.org/10.1006/fmic.1999.0332.

[77] Kiuchi, K., Watanable, S., 2004. Industrialization of Japanese Natto. Food Science and Technology (Newyork-Markel Dekker) 193–238.

[78] Y. Peng, Q. Huang, R.H. Zhang, Y.Z. Zhang, Purification and characterization of a fibrinolytic enzyme produced by *Bacillus amyloliquefaciens* DC-4 screened from douchi, a traditional Chinese soybean food. Comparative biochemistry and physiology, Part B, Biochem. Mol. Biol. 134 (1) (2003) 45–52, https://doi.org/10.1016/s1096-4959(02)00183-5.

[79] S. Dakwa, E. Sakyi-Dawson, C. Diako, N.T. Annan, W.K. Amoa-Awua, Effect of boiling and roasting on the fermentation of soybeans into dawadawa (soy-dawadawa), Int. J. Food Microbiol. 104 (1) (2005) 69–82, https://doi.org/10.1016/j.ijfoodmicro.2005.02.006.

[80] E.N. Dike, S.A. Odunfa, Microbiological and biochemical evaluation of a fermented soybean product-soy-dawadawa, J. Food Sci. Technol. 40 (2003) 606–610, https://doi.org/10.1080/10826068.2018.1550656.

[81] L.I. Ouoba, C. Parkouda, B. Diawara, C. Scotti, A.H. Varnam, Identification of *Bacillus spp.* from Bikalga, fermented seeds of *Hibiscus sabdariffa*: phenotypic and genotypic characterization, J. Appl. Microbiol. 104 (1) (2008) 122–131, https://doi.org/10.1111/j.1365-2672.2007.03550.x.

[82] L.I.I. Ouoba, B. Diawara, W.K. Amoa-Awua, A.S. Traoré, P. Lange Møller, Genotyping of starter cultures of *Bacillus subtilis* and *Bacillus pumilus* for fermentation of African locust bean (*Parkia biglobosa*) to produce Soumbala, Int. J. Food Microbiol. 90 (2004) 197–205.

[83] S. Fincan, B. Enez, S. Özdemir, F. Matpan Bekler, Purification and characterization of thermostable a-amylase from thermophilic *Anoxybacillus flavithermus*, Carbohydr. Polym. 102 (2014) 144–150.

[84] A. Simair, I. Khushk, A. Qureshi, M. Bhutto, H. Chaudhry, K. Ansari, C. Lu, Amylase production from thermophilic *Bacillus sp*. BCC 021-50 isolated from a marine environment, Fermentation 3 (2) (2017) 25, https://doi.org/10.3390/fermentation3020025.

[85] T.N. Almanaa, P. Vijayaraghavan, N.S. Alharbi, S. Kadaikunnan, J.M. Khaled, S. A. Alyahya, Solid state fermentation of amylase production from *Bacillus subtilis* D19 using agro-residues, J. King Saud Univ. Sci. 32 (2020) 1555–1561, https://doi.org/10.1016/j.jksus.2019.12.011.

[86] J.S. Paul, E. Beliya, S. Tiwari, K. Patel, N. Gupta, S.K. Jadhav, Production of biocatalyst α-amylase from agro-waste 'rice bran' by using *Bacillus tequilensis* TB5 and standardizing its production process, Biocatal. Agric. Biotechnol. 101648 (2020), https://doi.org/10.1016/j.bcab.2020.101648.

[87] T. Paul, A. Das, A. Mandal, S.K. Halder, P.K. Das Mohapatra, B.R. Pati, K. C. Mondal, Biochemical and structural characterization of a detergent stable alkaline serine keratinase from *Paenibacillus woosongensis* TKB2: a potential additive for laundry detergent, Waste Biomass Valorization 5 (2014) 563–574.

[88] A.A. Salim, S. Grbavčić, N. aŠekuljica, M. Vukašinović-Sekulić, J. Jovanović, S. J. Tanasković, Z. Knežević-Jugović, Enzyme production by solid-state fermentation on soybean meal: a comparative study of conventional and ultrasound-assisted extraction methods, Biotechnol. Appl. Biochem. 66 (3) (2019) 361–368, https://doi.org/10.1002/bab.1732.

[89] H.M. Abdel-Mageed, A.Z. Barakat, R.I. Bassuiny, A.M. Elsayed, H.A. Salah, A. M. Abdel-Aty, S.A. Mohamed, Biotechnology approach using watermelon rind for optimization of α-amylase enzyme production from *Trichoderma virens* using response surface methodology under solid-state fermentation, Folia Microbiol. 67 (2022) 253–264.

[90] L. Yu, T. Lei, X. Ren, X. Pei, Y. Feng, Response surface optimization of l-(+)-lactic acid production using corn steep liquor as an alternative nitrogen source by *Lactobacillus rhamnosus* CGMCC 1466, Biochem. Eng. J. 39 (3) (2008) 496–502, https://doi.org/10.1016/j.bej.2007.11.008.

[91] M. R. R. Rao, S.V. Raju, M.V.L.N. Reddy, P. Pavani, Interaction between dietary calcium and non-phytate phosphorus levels on growth, bone mineralization and mineral excretion in commercial broilers Anim. Feed Sci. Technol. 131 (1–2) (2006) 135–150, https://doi.org/10.1016/j.anifeedsci.2006.02.011.

[92] R. Muralidhar, S.N. Gummadi, V.V. Dasu, T. Panda, Statistical analysis on some critical parameters affecting the formation of protoplasts from the mycelium of *Penicillium griseofulvum*, Biochem. Eng. J. 16 (3) (2003) 229–235, https://doi.org/10.1016/s1369-703x(03)00024-x.

[93] Y.D. Obafemi, A.A. Ajayi, G.I. Olasehinde, et al., Screening and partial purification of amylase from aspergillus niger isolated from deteriorated tomato (*Lycopersicon esculentum* mill.) fruits, Afr. J. Clin. Exp. Microbiol. 19 (1) (2018) 47–57, https://doi.org/10.4314/ajcem.v19i1.7.

[94] K.V. Bhaskara Rao, K. Ashwini, K. Gaurav, et al., Optimization, production and partial purification of extracellular αamylase from *Bacillus* sp. *marini*, Arch. Appl. Sci. Res. 3 (1) (2011) 33–42.

[95] P. Tallapragada, R. Dikshit, A. Jadhav, et al., Partial purification and characterization of amylase enzyme under solid state fermentation from *Monascus sanguineus*, J. Genet. Eng. Biotechnol. 15 (2017) 95–100, 10.1016/j.jgeb.2017.02.003. PMid: 30647646.

[96] D. Raul, T. Biswas, S. Mukhopadhyay, et al., Production and partial purification of alpha amylase from *Bacillus subtilis* (MTCC 121) using solid state fermentation, Biochem. Res. Int. (568141) (2014) 1–5, https://doi.org/10.1155/2014/568141. PMid: 24672727.